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Review

Investigations of antimicrobial peptides in planar film systems

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Abstract

Planar systems – monolayers and films – constitute a useful platform for studying membrane-active peptides. Here, we summarize varied approaches for studying peptide organization and peptide–lipid interactions at the air/water interface, and focus on three representative antimicrobial membrane-associated peptides—alamethicin, gramicidin, and valinomycin. Experimental data, specifically surface pressure/area isotherms and Brewster angle microscopy images, provided information on peptide association and the effects of the lipid monolayers on peptide surface organization. In general, film analysis emphasized the effects of lipid layers in promoting peptide association and aggregation at the air/water interface. Importantly, the data demonstrated that in many cases peptide domains are phase-separated within the phospholipid monolayers, suggesting that this behavior contributes to the biological actions of membrane-active antimicrobial peptides.

Keywords: Langmuir monolayer; Brewster angle microscopy; Peptide–membrane interaction; Peptide film

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1. Introduction

The hydrophobic and amphipathic properties of many antimicrobial peptides (AMPs) generally dictate that they tend to exhibit high affinity to the phospholipid assemblies comprising the cell membrane [1], and that their structures are significantly modified in membrane environments compared to aqueous solutions [1,2]. In particular, the biological activities of AMPs are believed to be strongly related to their interactions with the cell membrane [3,4]. Elucidating the dynamical and structural characteristics of AMPs within lipid assemblies is thus highly important for understanding their mechanisms of action and biological effects.

Planar lipid systems, such as supported monolayers and films, have been a useful model for studying membrane interactions of antimicrobial peptides. Planar systems generally resemble the lateral organization of phospholipids in cellular membranes, and can, in fact, be perceived as better mimicking actual cell surfaces on a molecular scale due to their non-existent surface curvatures [5]. Utilization of planar lipid models for studying peptide–membrane interactions takes specific advantage of the availability of diverse analytical techniques for surface characterization, including microscopy methods [6], scattering [7], and thermodynamic analysis [8,9].

Langmuir monolayers deposited at the air/water interface of aqueous solutions have been particularly informative as models for studying peptide organization and membrane interactions of AMPs. The preferred localization of amphiphilic peptide molecules at the water surface and the presence of a defined interface constitute an environment in which membrane-active molecules, such as AMPs, can self-assemble. Particularly important for studying peptide–membrane interactions has been the deposition of *phospholipid* monolayers at the air/water interface. Phospholipid monolayers have been employed in varied investigations of peptide–membrane interactions, yielding information both on the cooperative properties of lipid-interacting peptides, as well as on the organization and disruption of the lipid layers following AMP interactions. In many instances, physical and morphological analyses of Langmuir film/AMP assemblies have been conducted by thermodynamic methods (pressure-area isotherms [10]) and microscopy techniques (fluorescence microscopy, Brewster angle microscopy [11–13]).

An example for the use of Langmuir monolayers for studying peptide–membrane interactions has been a systematic study of a series of bombolitins, bumblebee-venom peptides, and their synthetic analogs [14]. That investigation employed fluorescence microscopy to investigate the structural disruption and phase separation induced by the incorporated peptides within the phospholipid monolayers [14]. Other representative studies explored the relationships between the antibacterial activities and membrane interactions of several variants of the horseshoe crab antimicrobial peptide polyphemusin I, in which some of the analogues displayed divergent amphipathic properties [15]. Langmuir monolayer analysis, in which penetration of peptides into varied lipid monolayers was investigated through monitoring the change in surface pressure at the air/water interface, showed that the degree of negative charge on the membrane surface was closely related to bacterial membrane permeabilization. Importantly, binding of the peptides to monolayers composed solely of lipopolysaccharides (LPS) pointed to preferential peptide binding to the saccharide headgroups, and re-emphasized the significance of electrostatic interactions for promoting peptide binding onto the external leaflet of the bacterial membrane [15].

Other experimental approaches for studying AMP association with Langmuir monolayers were described. A synchrotron grazing incidence diffraction and X-ray reflectivity study of frog skin antimicrobial peptides belonging to the peptidyl-glycylleucine-carboxyamide (PGLa) family which were added to Langmuir monolayers was reported [16]. That study concluded that the peptides were fully interspersed within negatively-charged phospholipids but formed distinct domains in zwitterionic monolayers, confirming the hypothesis that AMPs can discriminate between the major phospholipid components of bacterial and mammalian membranes. Recent developments have enabled the application of surface-sensitive X-ray and neutron scattering techniques for characterization of molecule-thin peptide crystalline sheets constructed as Langmuir monolayers [7].

In this paper, we briefly review methodologies employed for studying peptide assemblies at the air/water interface and their interactions with lipid films, and discuss representative experiments carried out in our laboratory on the subject, in which we investigated three representative peptides: alamethicin, gramicidin, and valinomycin (Table 1). In particular, we discuss experiments in which films of AMPs and AMP/lipids were created and characterized using several microscopy techniques, particularly Brewster angle microscopy (BAM), atomic force microscopy (AFM) and fluorescence microscopy, with the goal of obtaining a better understanding of AMP organization and interactions with membranes. We also describe the construction of a new chromatic phospholipid/diacetylene platform [17] for studying peptide–membrane

Table 1
Characteristics of the peptides analyzed in this study

Peptide	Sequence	Conformation	Source	References
Alamethicin	acetyl-**UPUAUAQUVUGLUPVUUQQ-**phenylalaninol	α-helix	*Trichoderma viride*	[38,39]
Gramicidins	formyl-(L)**X**-**E**-(L)**A**-(D)**L**-(L)**A**-(D)**V**-(L)**V**-(D)**V**-(L)**W**-(D)**L**-(L)**Y**-(D)**L**-(L)**W**-ethanolamine	β-helix	*Bacillus brevis*	[53]
Valinomycin	(-(L)**V**-(D)**H**-(D)**V**-(L)**La**-)$_3$	Cyclic	*Streptomyces fulvissimus*	[61]

U–α-amino isobutyric acid, X–valine or isoleucine, Y–tryptophan (Gramicidin A (Gr A)), or phenylalanine (Gr B), or tyrosine (Gr C), H–hydroxy-isovaleric acid, La–lactic acid.

interactions through analysis of colorimetric and fluorescence transformations.

2. Analytical methods for investigating peptide organization at the air–water interface

2.1. Thermodynamic analysis

Thermodynamic profiling of peptides at the air/water interface generally aims to provide information on the organization, phase transitions, and molecular interactions within the films. Such experiments are based on the recording and analysis of the surface-pressure (π)/area (A) isotherms of the peptide films. The different regions of the isotherms carry valuable information on film properties. Specifically, *changes in film organizations* give rise to the plateau regions in the π–A isotherms curve ($[\partial\pi]/[\partial A]=0$), in which more than one phase coexist in the film [18]. These transition regions, characterized by changes in the monolayer area without changes in the surface pressure, connect the stable phase regions that give rise to abrupt increases in the surface-pressures.

Interactions and association of peptides with Langmuir lipid monolayers have been characterized. Two main experimental techniques have been employed for thermodynamic analyses of peptide–lipid interactions at the air/water interface (Fig. 1). The first approach is based on the compression of *mixed peptide/lipid monolayers* while recording the *surface-pressure/area isotherms* (Fig. 1A). The isotherms carry information on the organization of both peptide and lipid components, and are generally analyzed by using the two dimensional Crisp phase rule [19], or the additivity rule [12] which provide information on the interactions and the extent of miscibility among the film constituents. Thermodynamic parameters related to the mixing (excess free energy ΔG_{xs}, entropy ΔS_{xs} and enthalpy ΔH_{xs} of mixing) can be calculated as well [10].

Beside compression of as-prepared peptide/lipid monolayers, another approach involves the injection of peptides into the subphase underneath pre-assembled lipid monolayers and monitoring their adsorption into the film (Fig. 1B). Characterization of peptide insertion into the lipid film either relies on monitoring the time-dependent changes of surface pressure while keeping the film area constant [20], or alternatively keeping the *surface pressure* constant and recording the changes in molecular surface area as the amphiphilic peptide inserts into the lipid film [21]. Such adsorption experiments are based on the fact that AMPs, being mostly amphiphilic molecules, are at higher energy state in the bulk water phase compared with their adsorbed state at the air/water interface.

Investigations of peptide adsorption were pioneered by Shulman et al. [22,23] who identified three consecutive processes occurring when proteins associate with lipid films: adsorption, penetration, and solubilization. Previous studies have particularly emphasized the important roles in the adsorption and desorption processes of electrostatic and van der Waals attraction forces between the peptide and lipid molecules. These parameters have been intensively investigated [24–27] and led to development of a general approach for interpretation of adsorption isotherms [9,24–27]. In particular, the adsorption and penetration processes are believed to be closely dependent on peptide conformation, charge, amphipathicity, the compositions of the subphase and lipid films, and the initial surface pressure. The thermodynamic factors influenced by peptide adsorption onto lipid monolayers include the *total surface pressure change* ($\Delta\pi$) upon adsorption of the peptide molecules, the *rate of pressure change* ($\Delta\pi/\Delta t$), and the *exclusion pressure* (π_{ex}) in which the peptide no longer penetrates into the lipid monolayer but rather forms distinct aggregates at the air/water interface.

2.2. Microscopy analyses

The flat topology of films, in general, presents an important advantage in their study over vesicle assemblies. This flat morphology makes the film particularly accessible for various microscopic techniques, both in situ (at the air/water interface), or following film deposition onto solid substrates. An in situ microscopy technique gaining prominence in film analysis is Brewster angle microscopy (BAM) [28,29]. BAM is based on the physical phenomenon that when a polarized light is shone on a refractive surface such as water, the angle of incidence affects the ratio between the reflected and refracted beams differently for p and n polarizations [29]. Thus, at a particular incidence angle θ, ($\tan\theta=n_1/n_2$, where n_1,n_2 are the refractive indices of air and water, respectively), called the Brewster angle, the reflected light has no component of the electric vector in the plane of incidence (p-polarized) (Fig. 2A). BAM mounted on the Langmuir trough allows visualization of film

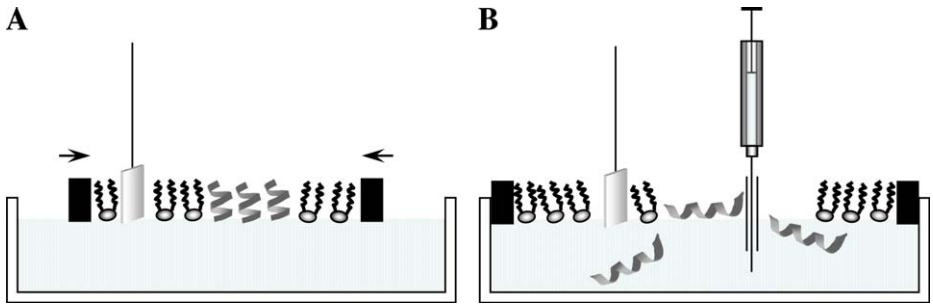

Fig. 1. Two approaches for studying peptide–lipid interactions at the air–water interface: (A) Cooperative compression of mixed films. (B) Injection of peptide solution beneath the lipid monolayer.

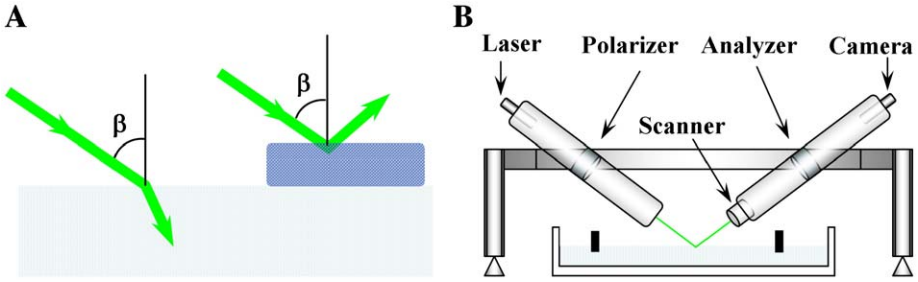

Fig. 2. (A) Polarized light interaction with water surface and a deposited film at the Brewster Angle (β). (B) A schematic description of a Brewster Angle Microscope (BAM).

organization, nucleation, segregation, and anisotropy of microscopic domains within the film [12,13].

Other microscopy techniques can be applied after transferring the films from the air/water interface onto different solid support (glass, mica, silicon) using Langmuir–Blodgett or the Langmuir–Schaefer techniques [30,31]. The immobilized films are then amenable to high-resolution surface imaging techniques such as atomic force microscopy (AFM), which enables submicron mapping of film surfaces. AFM can produce high-resolution topographical information on the film surface from which molecular ordering and distribution can be derived. AFM revealed, for example, molecular alignment and domain organization of AMPs within *E. coli* membrane–lipid monolayers [32].

Fluorescent microscopy has been employed for illumination peptide association in lipid films through doping the films with fluorescent probes [13,33]. Such markers can be physically incorporated inside the film, or covalently attached to phospholipid molecules comprising the monolayers. The microscopy experiments could image structural features on the film surface induced by peptide interactions. Fluorescent microscopy revealed, for example, the formation of condensed domains of the AMP alamethicin in lipid films—apparent through "squeezing out" of the fluorescent probes distributed within the monolayers [13]. In contrast, interaction of protegrin-1 with phospholipid monolayers yielded an increase of the disordered liquid phase resulting in diffusion of the probe throughout the monolayer surface [33].

Other techniques have been applied for structural characterization of membrane-active peptides in films. FTIR analysis of mixed peptide/lipid monolayers shed light on the conformation [34] and orientation [11] of AMPs in the films. Neutron diffraction [35,36], X-ray reflectivity (XR) [37], and grazing-incidence X-ray diffraction (GIXD) [16] have focused on the *lipid monolayers*, elucidating the effects of membrane-associated peptides on the structures and organization of the films.

3. Investigations of antimicrobial peptides in film environments

3.1. Alamethicin

Alamethicin is a 20 amino acid antibiotic peptide (Table 1), produced by the fungus *Trichoderma viride* [38,39]. It adopts α-helical structure in hydrophobic environments and displays high affinity to phospholipid bilayers. The peptide is believed to form voltage-gated ion channels in membranes according to the "barrel-stave" model [40,41]. Recently, pore formation induced by two-dimensional aggregation of alamethicin at the phospholipid–water plane was proposed [42]. Langmuir monolayers have been employed for investigation of alamethicin association with lipid assemblies at air–water interface [13,37,42]. Compression of films comprising pure alamethicin on a water subphase indicated the formation of solid peptide monolayers with an area per molecule of 320 $Å^2$ and the α-helix axis oriented parallel to the air/water interface [42].

We have investigated interactions and association properties of alamethicin within different films at the air/water interface by surface-pressure/area isotherms and through application of BAM and confocal fluorescence microscopy [13]. Fig. 3A depicts the surface-pressure area isotherm of alamethicin on a water subphase. The isotherm exhibits the expected threshold at a molecular area of approximately 320 $Å^2$ corresponding to the projected area of an alamethicin α-helix oriented parallel to the water surface [37,42]. The steep rise of the isotherm is followed by a plateau region at a surface pressure of approximately π=33 mN/m, indicating that at higher surface pressures the peptide monolayer collapse and form multilayers rather than reorient into compressed vertical helical positions [37,42].

Application of BAM revealed remarkable diversity of alamethicin aggregates at different points along the compression curve [13] (Fig. 3B). The appearance of the "butterfly" shapes and irregular rectangles was reproducible, and might be related to different nucleation rates of the dissolved alamethicin [43,44]. The intriguing BAM image recorded close to the molecular area threshold of 320 $Å^2$ points to the onset of physical contact among the separate alamethicin domains (Fig. 3Bii). The different tones of the individual domains are due to orientational anisotropy corresponding to different orientations of the peptide aggregates. This result is consistent with ordering of the helical peptides parallel to the water surface [37,42], and is in agreement with previously published AFM data of alamethicin films transferred to glass substrates showing one-dimensional crystalline structures within the peptide monolayers [37]. Specifically, helices deposited in parallel to the air–water interface are co-aligned and form anisotropic two-dimensional crystalline assemblies. Their interaction with the BAM polarized laser light gives rise to birefringence which is manifested in different contrasts of the domains, according to their orientation with respect to the polarization plane.

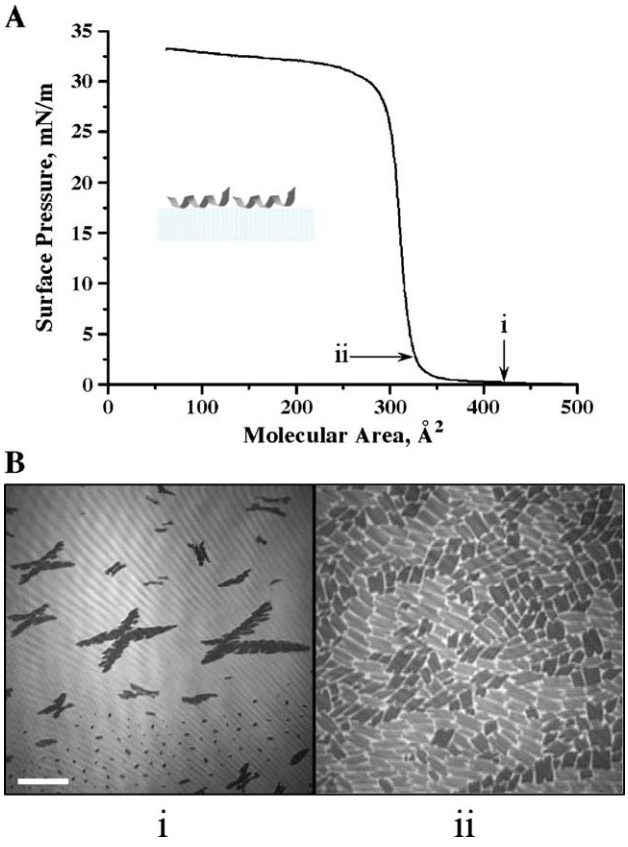

Fig. 3. (A) Surface pressure-area isotherm of an alamethicin monolayer, recorded at 25 °C. The insert depicts the proposed peptide conformations at the respective phases. (B) BAM images recorded in the indicated points within the isotherm. The scale bar represents 50 μm.

Adsorption experiments designed to evaluate alamethicin migration from the aqueous sub-phase into the air–water interface indicated the existence of a concentration threshold, *below* which no surface pressure increase could be detected [13]. Such a concentration threshold reflects the minimal concentration of alamethicin in the water subphase that facilitates peptide aggregate formation, diffusion to the air/water interface and sufficiently significant interactions among the peptide domains [45–47].

Additional experiments were carried out to explore alamethicin interactions with *phospholipid monolayers* (Fig. 4). Fig. 4A demonstrates that the presence of a dimyristoylphosphatydilcholine (DMPC) monolayer at the air/water interface promoted peptide adsorption, showing instant surface pressure increase at much *lower* peptide subphase concentrations compared to the situation of pure alamethicin films on water. An exclusion pressure of 27.5 mN/m was observed in the adsorption isotherms [13]. This value echoes the surface pressure in which collapse onset was apparent in the pure alamethicin film compressed on water (Fig. 3A). This result suggests that *phase-separated* alamethicin domains are formed within the phospholipid monolayer following peptide adsorption.

Indeed, a central question we addressed in the experiments was whether, and at what conditions, alamethicin is *miscible* within phospholipid films deposited at the air/water interface, or whether it aggregates in distinct domains. Fig. 4B depicts BAM images of a DMPC film deposited over an aqueous solution into which alamethicin was injected. Discrete peptide domains were clearly apparent in the image, confirming the *immiscibility* of alamethicin within the DMPC monolayer. This result is consistent with previous studies indicating that alamethicin was phase-separated within anionic, cationic, and zwitterionic lipids and formed separated domains of defined shapes in such film environments [13,37,42]. Immiscibility of alamethicin was ascribed to the hydrophobic mismatch between the peptide and the lipid layers, and might also explain its propensity to oligomerize and form intact ion channels [4,48,49].

Consistent with the above observations, recent monolayer experiments under electric field and X-ray reflectivity have discovered stable phase-separated aggregates of alamethicin within lipid films, oriented horizontally with respect to the air/water interface [37,42]. These studied also identified a high energy barrier for peptide reorientation from the parallel to a perpendicular orientation with respect to the lipid/water interface, and led to proposal of a new asymmetrical "lipid-covered ring" model of the voltage-gated ion channel of alamethicin [37,42]. These data appear different than previous studies employing lipid bilayers in which predominantly transmembrane organization of the peptide was observed [50–52]). This discrepancy might indicate that at the air–water interface the helix–dipole interactions keep the peptide in an

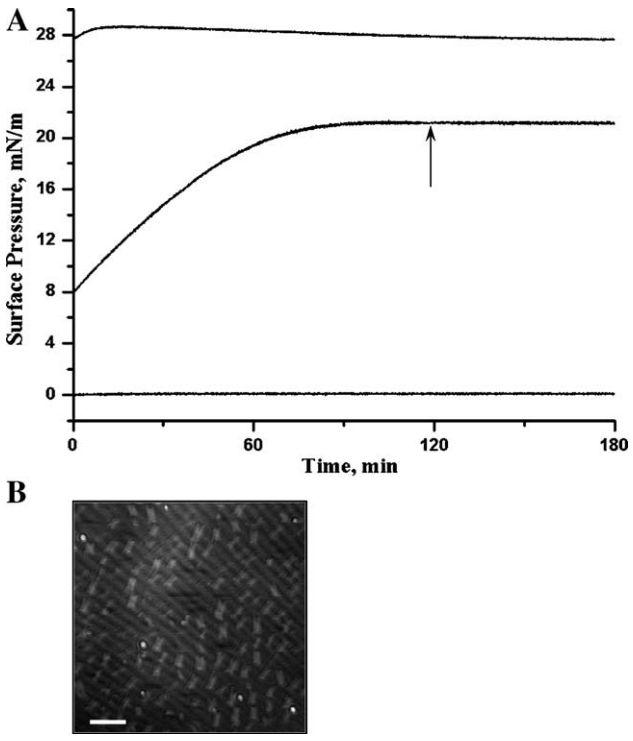

Fig. 4. (A) Adsorption isotherms of alamethicin (5.0×10^{-8} M subphase concentration) and DMPC monolayers at initial surface pressures π_{in} of 8 and 28 mN/m (shown for comparison also the adsorption isotherm of alamethicin and *pure* water—bottom curve at zero surface pressure). (B) A BAM image recorded at the indicated point within the adsorption isotherm. The scale bar represents 50 μm.

orientation parallel to the surface, whereas the dipole charges are screened in a water-bilayer–water layer.

3.2. Gramicidin

The linear peptides gramicidin A, B, and C comprise a family of small hydrophobic antimicrobial peptides produced by the soil bacterium *Bacillus brevis*. [53] Valine-gramicidin A (VGA), the most widely-studied member of this AMP family, is a 15-residue β-helical peptide (Table 1) which forms dimers in a head-to-head fashion resulting in membrane-spanning ion channels that are selective for monovalent cations such as H^+, Tl^+, NH_4^+ and the alkali metals [54]. Gramicidin A was found to adopt both single-stranded and double-stranded conformations having right or left-handed helices in different environments [55,56].

A surface-pressure/area isotherm (20 °C) of a natural mixture of gramicidins (consisting mostly of gramicidin A [34]) shown in Fig. 5A points to a different compression profile of gramicidin as compared to alamethicin (Fig. 3 discussed above). Specifically, the isotherm features a *phase transition* at approximately 20 mN/m, rather than a single stable phase observed for alamethicin. A single-stranded to double-stranded transition was proposed, and an alternative picture of multilayer formation has been also suggested [57,58]. The divergent behavior of the two peptides can most likely be traced to their different amino-acid sequences, polar/nonpolar area distribution, and distinct secondary structures. Importantly, the above data indicate that differences in structures probably directly affect the surface properties of the peptides.

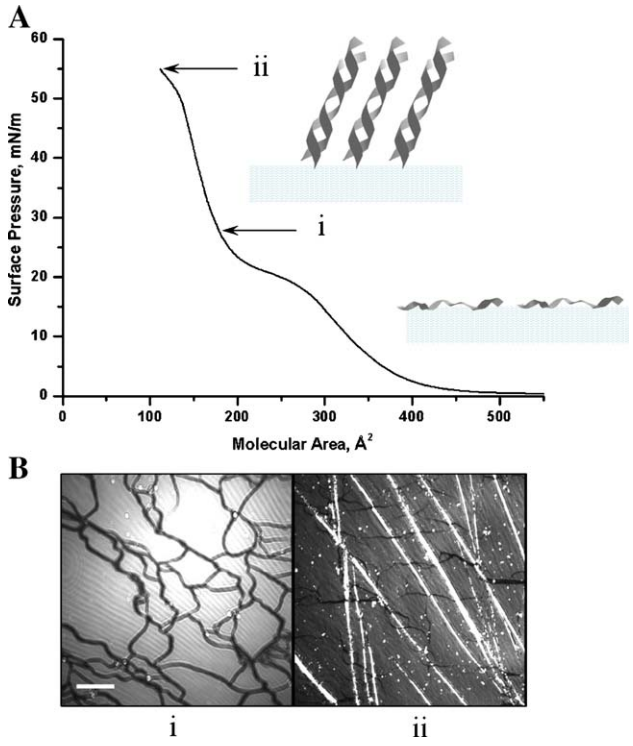

Fig. 5. (A) Surface pressure-area isotherms of gramicidin (natural mixture) at 20 °C. The inserts depict the proposed peptide organization at the respective phases. (B) BAM images of the gramicidin monolayer at the indicated points. The scale bar represents 50 μm.

BAM analysis (Fig. 5B) aids in further elucidating the surface phenomena occurring in gramicidin films. Formation of solid, homogeneous gramicidin monolayers subsequent to the phase transition was clearly observed (Fig. 5Bi). Visualization of the film was aided by repeated recompression, which induced abundant "fractures" within the film surface (Fig. 5Bi). These extraordinary straight-line deformations appeared at the approximately 50 mN/m collapse pressure (Fig. 5Bii). The pronounced brightness of the fractures suggests the formation of peptide *multilayers*. This interpretation is consistent with electron micrographs of collapsed gramicidin films, in which the height of the elevated domains was estimated to be approximately 40 Å [59].

Additional details concerning the structural aspects of gramicidin films at the air/water interface were provided by other analytical techniques. A diffraction and polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS) study has shown that during the pressure-induced phase transition of the gramicidin film, its thickness abruptly increased from ~6–9 Å to ~25 Å, ascribed to a transformation of the peptide from unfolded to an intertwined double-stranded $\beta^{5.6}$-helical conformation oriented at 60° to the water surface [58]. This type of ordering transition might be reflected in the BAM experiment (Fig. 5Bi); the gramicidin film was visible *only after* the phase transition, indicating a disordered liquid phase in the initial compression stages.

Another detailed thermodynamic analysis pointed to the significance of the tryptophane residues in determining the surface ordering of gramicidin films [57]. Specifically, substitution of the tryptophane residue at position 9 by phenylalanine led to dramatic decrease in the molecular area at low surface pressures, and to almost complete disappearance of the phase transition. Also, replacement of the tryptophane at position 11 resulted in partial loss of the curve deflection. It was further suggested that trp–trp stacking interactions are involved in phase transition and molecular stabilization [57].

The interaction of gramicidin with phospholipid monolayers deposited at the air/water interface was studied by compression analysis and BAM (Fig. 6). The surface-pressure/area isotherm of mixed gramicidin/DMPC (1:1) film (Fig. 6A) exhibits the characteristic shoulder previously observed in compression experiments of pure gramicidin (Fig. 5A). Moreover, the phase transition at around 20 mN/m is at a similar surface pressure as pure gramicidin, which suggests that the presence of the phospholipid monolayer does not alter the fundamental properties of the gramicidin film.

BAM images depicted in Fig. 6B confirmed the immiscibility of gramicidin within the phospholipid film. Essentially, at high peptide concentrations around 50% (mole ratio within the film), the phospholipid molecules formed segregated domains distinguished as dark domains (due to the liquid-expanded phase of the lipids) within the highly reflective gramicidin film (Fig. 6B). Other studies have detected partial peptide miscibility within phospholipid films at mid-range peptide concentrations (5–50%) [60]. As an example, AFM

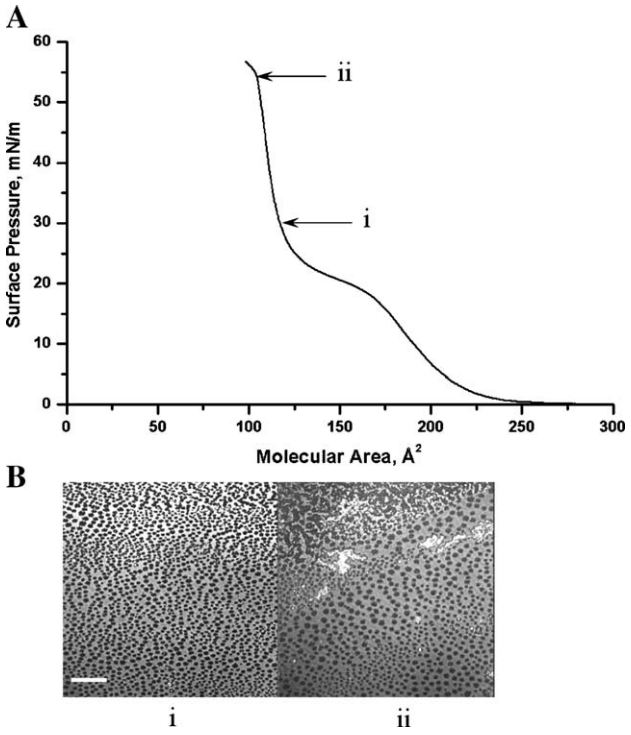

Fig. 6. (A) Surface pressure-area isotherms of mixed gramicidin/DMPC (1:1) film at 20 °C. (B) BAM images of the film at the indicated points. The scale bar represents 50 μm.

experiments have recorded the formation of "doughnut-shape" structures of gramicidin aggregates in dipalmitoylphsphatidylcholine (DPPC) monolayers, appearing at low peptide concentrations (<4%).

3.3. Valinomycin

Valinomycin is a cyclic depsipeptide ionophore produced by *Streptomyces fulvissimus* [61] (Table 1), which preferably binds and transport potassium cations across the cell membrane [62,63]. Cation transport is believed to be carried out through complexation by valinomycin through the six acid carbonyl oxygens of the peptide ring.

The surface-pressure/area isotherm of valinomycin on pure water [Fig. 7A, solid curve] reflects a liquid-expanded peptide film at lower surface pressures, and displays a film collapse at 30 mN/m. The extrapolated molecular surface area of approximately 350 Å/molecule is consistent with a previously published range of 320–370 Å/molecule, suggesting that the valinomycin rings are positioned parallel to the air/water interface [64–66].

BAM analysis provided additional details on the organization of the valinomycin molecules at the air/water interface. The liquid-expanded valinomycin film prior to the onset of collapse was essentially "invisible", which is expected due to the absence of organized film formation at the low surface pressure range. However, following film collapse at a surface pressure of approximately 30 mN/m, the BAM image reveals numerous bright dots (Fig. 7Bi) corresponding to valinomycin aggregates. Upon further compression, the valinomycin domains seemed to coalesce at a central region of the film (Fig. 7Bii). This phenomenon was reproducible, and might be ascribed to the effect of the *direction* of film compression towards the center region between the two barriers.

Distinctly different interactions and lateral organization of valinomycin were observed when the peptide was compressed on an aqueous sub-phase containing K^+ ions. The surface pressure-area isotherm of valinomycin deposited on a 1.0 M KCl solution, depicted in the broken curve in Fig. 7A, exhibited two additional phase transitions compared to the corresponding isotherm on pure water. The first transition occurred at approximately 23 mN/m and was found to be strongly dependent on the concentration of potassium ions in the subphase [64,66]. This transition was assigned to the formation of a "solid" phase constituting valinomycin molecules adopting rigid "bangle" conformations upon potassium complexation

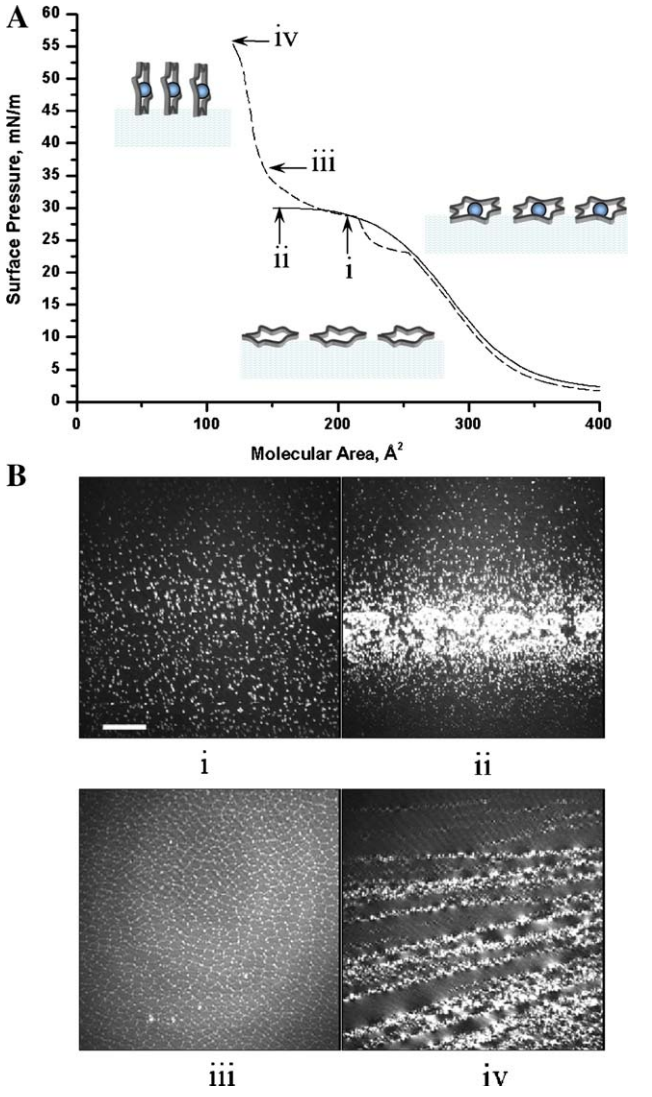

Fig. 7. (A) Surface pressure-area isotherm of valinomycin on water (solid line) and on 1.0 M KCl solution (dashed line). The inserts depict the proposed peptide organization at the indicated phases. (B) BAM images of valinomycin monolayer on water, (i) and (ii); and on 1.0 M KCl solution, (iii) and (iv). The scale bar represents 50 μm.

[65,67]. Indeed, valinomycin is known to lose its structural flexibility and transforming into defined structures upon complexing potassium [65].

The surface pressure of the second transition at approximately 30 mN/m was found to be *independent* of the subphase potassium concentration, suggesting that the valinomycin does not bind additional potassium ions following the transition. However, the average extrapolated molecular area of the third phase (around 180 A^2/molecule in Fig. 7A) was found to be highly sensitive to potassium concentration. [66] indicating that potassium ions interfere with the reorientation of "bangle"-shaped molecules from parallel to perpendicular position with respect to the water surface [67].

Intriguingly, previous studies have shown that the shape of the valinomycin isotherm was also dependent upon the *negative counter-ion* as well. Specifically, it was discovered that the first transition entirely disappeared when potassium-*fluoride* was present in the water subphase, and the surface pressure of the transition strongly varied when bromide, chloride or iodide were added [67]. These results might be related to the relationship between the size of the ions and the space of the valinomycin cavity, and consequent disruption of the formation of the bangled conformation [65].

The presence of potassium ions in the subphase clearly affected the structural features of the valinomycin monolayer, as apparent from the BAM data (Fig. 7Biii, iv). Specifically, after the second phase transition of the compressed valinomycin film (Fig. 7Biii) the BAM image featured interspersed polygonal peptide domains, suggesting long-range interactions among the valinomycin aggregates. The contribution of longer-range ordering was also apparent in the BAM image recorded after film collapse at approximately 50 mN/m (Fig. 7Biv), which shows straight solid valinomycin arrays. The bright appearance of the valinomycin assemblies indicates multilayer formation and resembles the peptide behavior on pure water subphase (Fig. 7Bii). This result suggests a propensity of the compressed solid peptide domains for anisotropic aggregation at the air/water interface.

The ion-complexing and membrane transport properties of valinomycin have fostered extensive research on mixed valinomycin–lipid film systems. Such films have been employed both for exploring the fundamental aspects of the ion transport phenomena, and also as a basis for creation of new ion-sensing systems. In particular, the effect of varying the lipid environment upon valinomycin properties was apparent in different film studies [68]. For example, while miscibility was observed in mixed monolayers of valinomycin and stearic acid [68], phase separation in almost all mole-ratios was found in case of mixed films of valinomycin and egg-lecithin [64] or arachidic acid [69].

The surface-pressure/area isotherm of an equimolar valinomycin/DMPC film is depicted in Fig. 8A. The overall shape of the compression isotherm of the valinomycin/lipid mixture (Fig. 8A) is similar to the isotherm of the pure peptide (Fig. 7A). In addition, the phase transition of the peptide/lipid film at approximately 30 mN/m is close to the value recorded for pure valinomycin. Taken together, the comparison between the

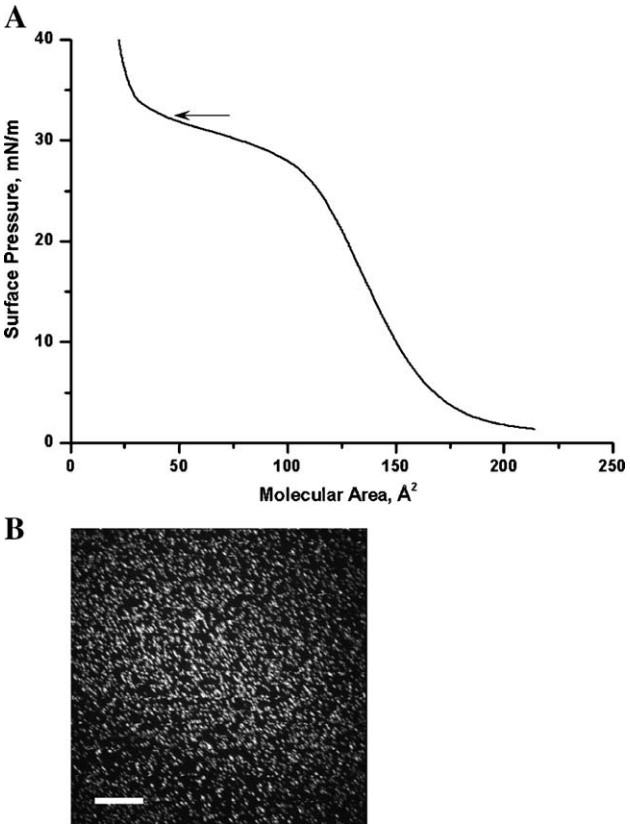

Fig. 8. (A) Surface pressure-area isotherm of a mixed valinomycin/DMPC (1:1) monolayer on water. (B) A BAM image recorded at the indicated point within the isotherm. The scale bar represents 50 μm.

thermodynamic data of the pure peptide and the peptide/lipid film suggests that, at the equimolar 1:1 peptide/lipid ratio employed, valinomycin is most likely *phase-separated* in the phospholipid monolayer. Indeed, the BAM image shown in Fig. 8B corroborates this conclusion, depicting an abundance of interconnected bright valinomycin domains deposited within the dark phospholipid background.

Lipid association was also shown to significantly affect the ion-complexation capabilities of valinomycin. Specifically, the ability of valinomycin to bind potassium ions was intimately dependent upon the *surface charge* of the lipid monolayers [70]. That study demonstrated that no ion binding occurred when valinomycin was mixed with positively-charged lipids, while the presence of negatively-charged lipid domains promoted the formation of potassium/valinomycin complexes [70]. These results suggest that the lipid environment, and specifically domain boundaries in the films, play important roles in determination of the ion transport properties of valinomycin.

4. Peptide interactions with lipid/polydiacetylene films

Diacetylenic lipids, first synthesized by Wegner in 1969 [71], have attracted considerable interest due to the unique chromatic properties of their polymerized form, polydiacetylene (denoted PDA, Fig. 9) [72]. Specifically, closely packed diacetylene lipid monomers can undergo topotactic polymerization by ultraviolet irradiation, yielding aligned PDA

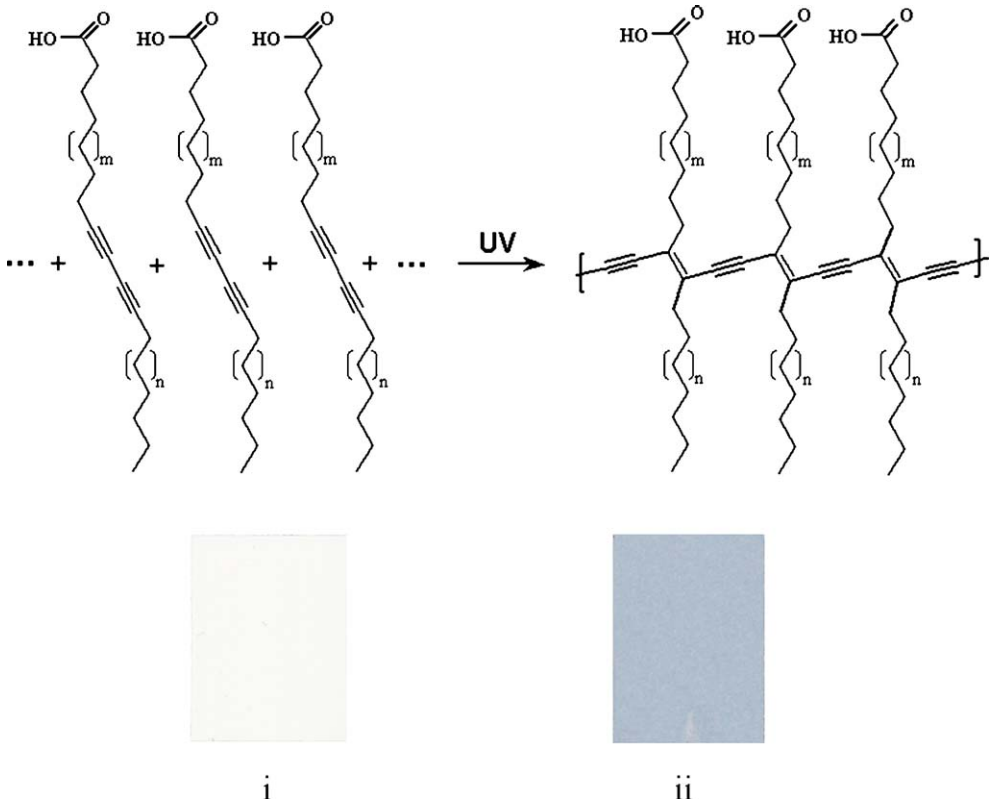

Fig. 9. Schematic picture depicting the polymerization of diacetylene carboxylic acid. Shown also are the visible colors of the film: (i) non-polymerized film; (ii) polymerized diacetylene film. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assemblies which appear intensely blue to the eye due to the $\pi \rightarrow \pi^*$ absorption of the conjugated backbone (Fig. 9) [71]. Furthermore, blue PDA can undergo a structural transition, shortening the effective π-conjugated length, and yielding a red-phase polymer [73]. The blue-red transitions of PDA can be induced by varied external factors such as temperature [74], pH changes [75], and mechanical compression [76], and have been observed both in vesicle systems [77], and in thin films [78].

We have recently demonstrated that mixed films of phospholipids and PDA can be utilized for studying membrane phenomena such as cooperativity in lipid organization [17], and peptide–membrane interactions [79]. In that context, our experiments supported the notion that phospholipid/PDA mixed films could be perceived as bio-mimetic membrane assemblies that further constitute a chromatic platform for studying membrane phenomena. In particular, the experiments indicated that films can be created with segregated phospholipid and diacetylene domains that are interspersed within the film, and can clearly interact and mutually affect their structure and organization [17]. Fig. 10, for example, depicts BAM images of DMPC/diacetylene films containing different concentrations of DMPC and diacetylene. The bright structures correspond to the diacetylene multilayers formed through film compression, interspersed within the lipid background. Importantly, the microscopy images clearly show that significantly different diacetylene shapes appear within the mixed films, depending on

Fig. 10. BAM images recorded at 18 °C of mixed diaceylene/DMPC films with different mole rations: (i) pure diacetylene; (ii) diacetylene/DMPC (7:3 mole ratio); diacetylene/DMPC (1:1 mole ratio). The scale bar represents 50 μm.

the relative concentrations of phospholipids in the Langmuir monolayer.

We have studied the interactions of antimicrobial peptides with the mixed lipid/diacetylene films (Figs. 11 and 12), and further investigated whether *polymerized* diacetylene (polydiacetylene, or PDA) can be employed for chromatic sensing of peptide interactions with the bio-mimetic membranes. Fig. 11 depicts the adsorption isotherm and BAM images of mixed DMPC/diacetyene films following injection of alamethicin into the aqueous sub-phase. The adsorption isotherm (Fig. 11A) shows an immediate increase in surface pressure following peptide injection, indicating instantaneous migration of the peptide into the lipid/diacetylene film. The transition observed after 40 min at approximately 20 mN/m further indicates segregation and collapsing of the TRCDA monolayer into the trilayer organization, induced by the increased surface pressure exerted by alamethicin insertion.

BAM images shown in Fig. 11B provide additional information on alamethicin adsorption within the lipid/PDA film, and the structural consequences of this process. In particular, the microscopy analysis demonstrates that alamethicin preferably migrate into the *phospholipid* monolayer, rather than associating with the diacetylene matrix. Furthermore, alamethicin is not miscible within the lipids, but rather forms distinct microscopic domains (shown as the dark domains in Fig. 11B). These results imply that the presence of the diacetylene matrix does not affect the bio-mimetic nature of the phospholipid layer, and the association of the peptide with the phospholipids. While the thermodynamic and microscopy data indicate that diacetylene is not directly involved in alamethicin–lipid interactions, the BAM images clearly show that the gradual adsorption of the peptide in the film leads to formation of diacetylene multilayers—apparent as the bright domains in the images. The progressive growth of the diacetylene multilayers is due to increased surface pressure within the film following adsorption of the peptide [13], and point to the indirect effect of peptide–film interactions on the diacetylene.

Similar phenomena were observed in DMPC/diacetylene films to which valinomycin was added, Fig. 12. The surface-pressure/area isotherm in Fig. 12A shows a similar profile to the isotherm of a DMPC/diacetylene film without added peptide. BAM images recorded at different pressures along the isotherm help to elucidate the structural features of the valinomycin/DMPC/diacetylene film. Specifically, similar to the case of alamethicin adsorption depicted in Fig. 11B, the microscopy pictures in Fig. 12B indicate that valinomycin forms distinct domains at high pressure, shown as the bright spots randomly distributed in the phospholipid monolayer within the mixed film.

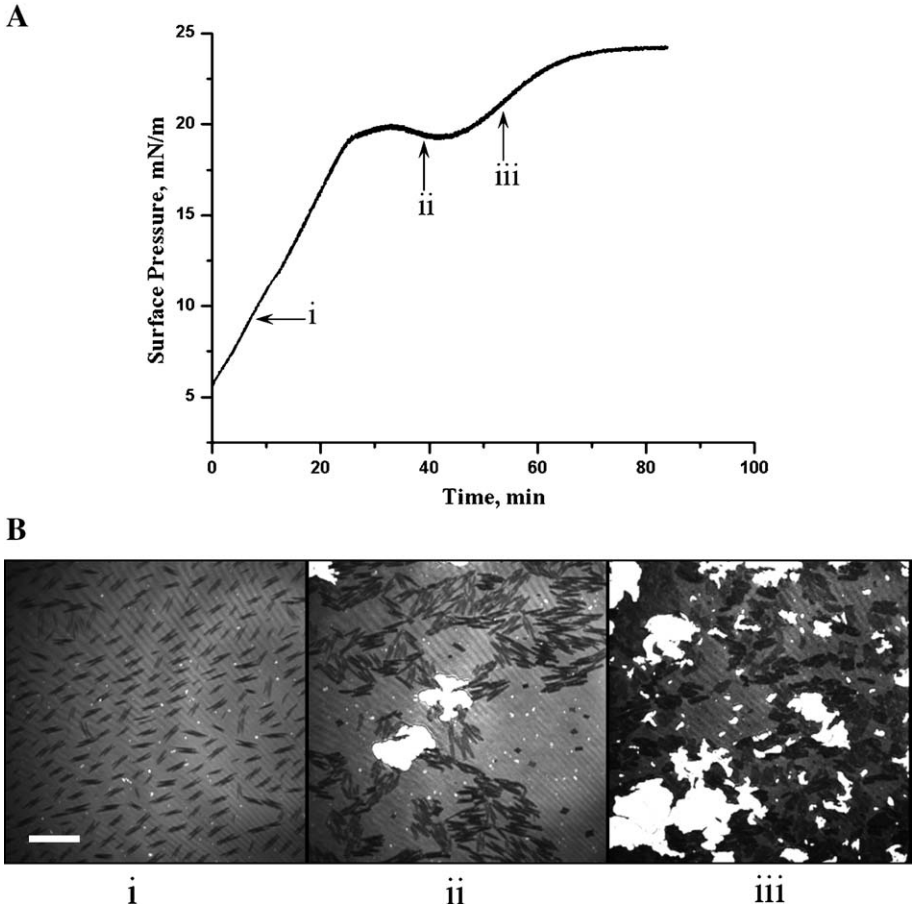

Fig. 11. (A) Adsorption isotherm of alamethicin onto the DMPC/diacetylene film (1:4 mole ratio) at 25 °C. (B) BAM images recorded at the indicated points on the adsorption isotherm. The scale bar represents 50 μm.

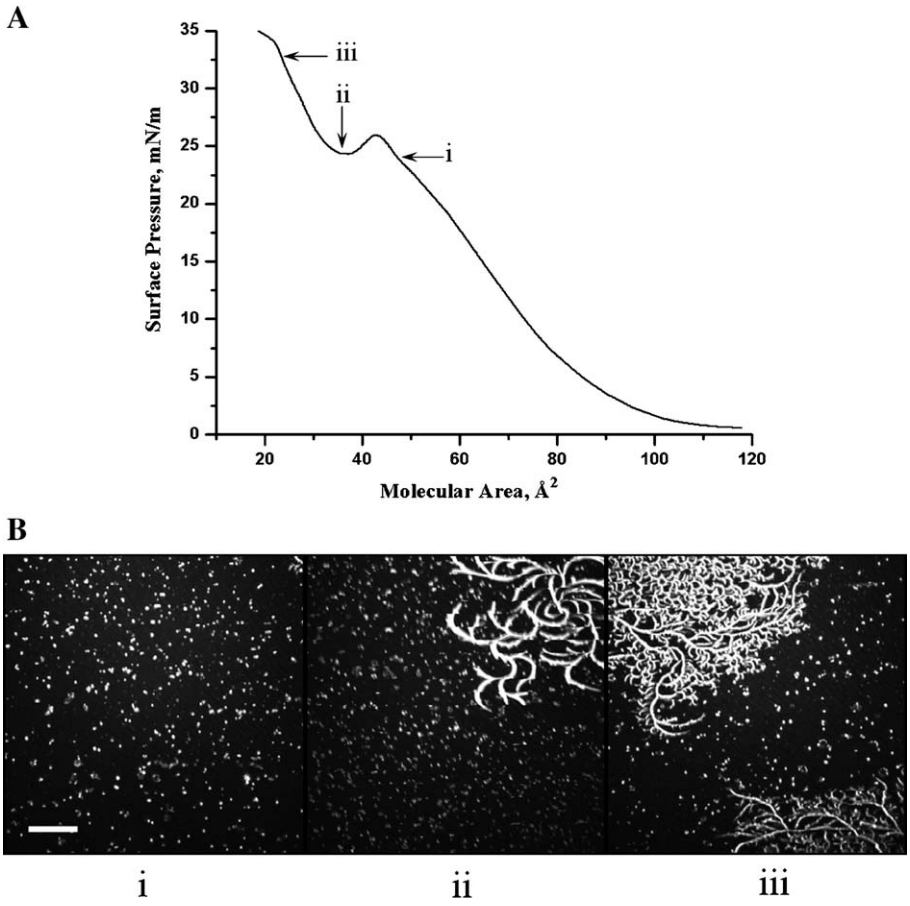

Fig. 12. (A) Surface pressure-area isotherm of DMPC/diacetylene/valinomycin (2/7/1) mixture on water at 25 °C. (B) BAM images recorded at the indicated points within the adsorption isotherm. The scale bar represents 50 μm.

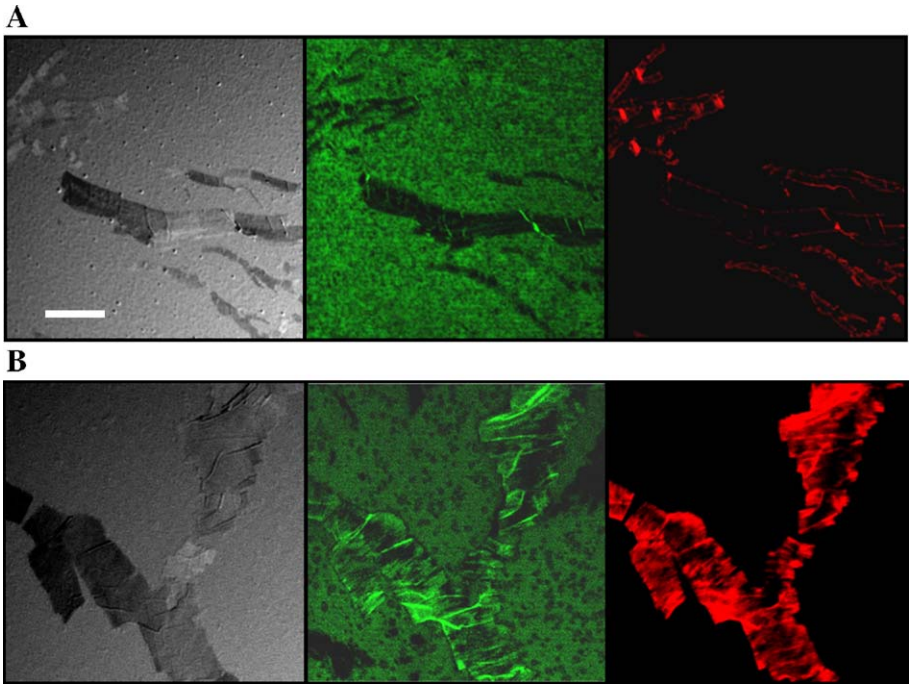

Fig. 13. Fluorescent images of polymerized NBD-PE/DMPC/diacetylene films (0.2:0.8:9 mole ratio) transferred to glass slides. (A) Addition of PBS buffer (1.0 mM) at pH 7.4. (B) Effect of injection of alamethicin at a concentration of 5.0×10^{-8} M in the PBS buffer. The scale bar represents 10 μm.

A noteworthy feature of the lipid/diacetylene films is their potential as colorimetric and fluorescence sensors for peptide–membrane interactions [79–81]. This feature is based on two important properties of the films. First, the mixed films can be transferred onto solid supports (using the Langmuir–Schaeffer technique) without altering the internal structure and organization of the film [17]. Second, polymerization of the lipid/diacetylene films, either prior to or after transfer of the films onto the solid support, results in the formation of blue polydiacetylene (PDA) which can then be exploited for sensing membrane interactions [13].

Fig. 13 depicts the remarkable fluorescent transformations observed in a glass-immobilized DMPC/PDA film following its interactions with alamethicin. The fluorescence confocal microscopy images in Fig. 13 correspond to a DMPC/PDA film incorporating also the fluorescent dye N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE), which co-localizes only within the *phospholipid* monolayer [13]. Fig. 13A shows the mixed film before peptide addition (only buffer added). The phase-contrast image of the NBD-PE/DMPC/PDA film (Fig. 13A, left panel) shows the distinct PDA domains within the phospholipid matrix. The fluorescence microscopy picture recorded using a 520 nm–550 nm band-pass filter emission (Fig. 13A, middle panel) displays only the fluorescence emitted from the NBD-PE marker, which as expected is distributed only in the phospholipid regions of the film. On the other hand, the emission image recorded by application of a >660 nm filter (Fig. 13A, right panel) depicts *only* the PDA fluorescence, which exhibits very low intensity—since the polymer is initially in the non-fluorescent blue phase. The faint fluorescence appearing at the edges of the polymer domains is most likely due to background structural perturbation induced at the lipid/PDA interfaces.

Dramatic transformations of the fluorescence distribution and intensities were apparent following addition of alamethicin to the NBD-PE/DMPC/PDA film (Fig. 13B). The numerous dark "islands" appearing within the NBD fluorescence background indicate that alamethicin forms distinct micro-domains only within the phospholipid monolayer (essentially ejecting the fluorescence marker from the peptide aggregates). Remarkably, the right panel in Fig. 13B shows a pronounced fluorescence signal (red) arising from the PDA domains (the image was recorded at 660 nm, which is the emission peak of the polymer). The fluorescence emission in the PDA regions was induced only after formation of the alamethicin domains, which affected the structural transformations of the polymer because of the increasing surface pressure within the mixed film [82].

5. Discussion

Our experiments as well as previous studies point to the close relationship between the behavior of membrane-active antimicrobial peptides at the air–water interface and their overall membrane interactions and association. The assembly characteristics of AMPs in particular are reflected in the film experiments. The propensity of alamethicin, for example, to self aggregate in membrane environments (forming oligomeric channel structures) was most likely the factor responsible for the spontaneous aggregation of alamethicin domains at the air–water surface even at zero surface pressure (Fig. 3). In contrast, gramicidin and valinomycin gave rise to liquid expanded phases, invisible by BAM system, up to surface pressures of 20 and 30 mN/m, respectively (Figs. 5 and 7), indicating much lesser tendency to aggregate. Indeed, the current model of valinomycin activity points to *single* molecules that ferry cations across the membrane. In the case of gramicidin, even though this peptide was proposed to form channels assembled by *two* peptide molecules [83], the interaction between the two units is in head-to-head fashion [83], rather than through lateral attraction. Indeed, the formation of visible bilayer structures following collapse of the gramicidin film (Fig. 5) points to the significance of the head-to-head interactions between gramicidin molecules in affecting their surface properties.

Ionic selectivity is also apparent in the monolayer experiments. Specifically, the high affinity of valinomycin to potassium cations gave rise to the pronounced change in the isotherm shape (Fig. 7A). The appearance of two additional phase transitions when K^+ ions were dissolved in the water subphase is another evidence for the significant conformational changes of the valinomycin molecules upon potassium binding.

The data summarized here emphasize the significance of analytical techniques, particularly BAM, in elucidating surface phenomena involving AMPs. BAM provided dramatic images of the peptide aggregates and their distribution within other film components. Significant enhancement of peptide adsorption to the phospholipid monolayers in comparison to the air–water interface was observed. In addition, indirect effects of the peptides on diacetylene moieties within mixed phospholipid/diacetylene films were observed. Formation of peptide micro-domains in such films was accompanied by an increase in surface pressure that in turn transformed the diacetylene into the multilayer phase state and eventually into the red-phase PDA. Significantly, the induction of the fluorescence transitions of the polymerized diacetylene in mixed phospholipid/PDA films can be implemented in biosensor constructs for the detection of specific membrane interactions.

6. Materials and methods

6.1. Materials

10,12-Tricosadiynoic acid (TRCDA) was purchased from GFS Chemicals (Powell, OH) and purified by dissolving the powder in chloroform, filtrating the resulting solution through a 0.45-μm Nylon filter, and evaporation of the solvent. Dimyristoylphosphatidylcholine (DMPC) [Avanti Polar Lipids, Alabaster, AL] was used as received. Construction of mixed DMPC/diacetylene films was achieved by dissolving DMPC and TRCDA (1:9 mole ratio) in chloroform at a total concentration of 2 mM.

The fluorescent probe, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) in HPLC-grade purity was purchased from Molecular Probes, Inc. (Eugene, Oregon USA) and was used as received. For making mixed NBD-PE/DMPC/diacetylene films NBD-PE was dissolved in chloroform to a concentration of

0.5 mM and was added to the DMPC/TRCDA solution to a final concentration of 2 mol%.

Alamethicin, gramicidin (natural mixture) and valinomycin were purchased from Sigma and was dissolved in ethanol at a concentration of 1.5 mg/ml.

All solvents were HPLC-grade pure.

The water subphase used in the Langmuir trough was doubly purified (18.3 mΩ resistivity) by using a Barnstead D7382 water purification system (Barnstead Thermolyne Corporation, Dubuque, IA-USA).

6.2. Surface-pressure/area isotherms

All surface pressure-area isotherms were measured with a computerized Langmuir trough (NIMA Model 611, Nima technology Ltd., Coventry, UK). The experiments were carried out at 25 °C using a thermostated Teflon film balance (7×50 cm^2). Surface pressure was monitored using a 1-cm-wide filter paper as a Wilhelmy plate. For each isotherm experiment, total of 70 nmol lipid mixture or 8.2 nmol peptide were spread on the water subphase (pH = 6.3). The film was allowed to equilibrate for 15 min after spreading. Compression was conducted at a constant barrier speed of 5 cm^2 min^{-1}. The surface pressure measurements were repeated at least three times, using a fresh mixture in each experiment. The isotherms shown in the figures represent three experimental runs, which were reproducible within ±5 Å^2·molecule^{-1}.

6.3. Adsorption of peptides from solution at the air/water and lipid/water interfaces

Adsorption experiments were carried out at 25 °C using a Nima 611 Teflon trough. Aliquots from the peptide solution were injected into the gently-stirred water subphase (total volume of 30 cm^2) through a short vertical tube, yielding different initial protein concentrations used in the experiments. The adsorption isotherms ($\Delta\pi$-time) of the peptides at constant air/water interfaces area (12 cm^2) were monitored using the Wilhelmy plate. The error in the measurements was ±0.5 mNm^{-1}.

In the experiments examining peptide penetration into lipid films the chloroform lipid solution was spread over the clean air/water interface and allowed to equilibrate for 20 min reaching the desired initial surface pressure (π_i). The peptides (dissolved in ethanol) were then injected close to the magnet stirrer, approximately 2 cm beneath the lipid monolayer. In case of peptide insertion into the DMPC/PDA films, initial compression of the film to 16 mN/m was carried out followed by polymerization of diacetylene by irradiation at 254 nm for 15 s. The water subphase underneath the film was separated by additional barriers, constructing an isolated chamber. The appropriate amounts of buffer (pH = 7.4) and peptide were then injected into the subphase.

6.4. Preparation of substrates for film deposition

Glass slides were dipped in a cleaning (piranha) solution consisting of 70 ml of H_2SO_4 and 30 ml of H_2O_2 for 30 min at 70 °C, followed by sonication in the same solution for 10 min., rinsed thoroughly with pure water and dried at 70 °C. Self-assembly of hydrophobic monolayers on the surface was carried out by immersion of the glass slide in 3.00 μl/ml OTS in cyclohexane solution for 12 h. Glass slides were then rinsed with cyclohexane to remove noncovalently bound OTS. In the fluorescence microscopy experiments, samples were prepared by transferring the polymerized DMPC/PDA films to the glass slides using the Langmuir–Schaefer method (horizontal touch method) [30,31].

6.5. Brewster angle microscopy

A Brewster angle microscope (NFT Co., Göttingen, Germany) mounted on a Langmuir film balance was used for in situ analysis of the films. The light source of the BAM was a frequency doubled Nd-YAG laser with a wavelength of 532 nm and 20–70 mW primary output power in a collimated beam. The BAM images were recorded with a CCD camera. The scanner objective was a Nikon super-long working distance objective with a nominal 10× magnification and diffraction-limited lateral resolution of 2 μm. The images were corrected in order to eliminate side-ratio distortion originating from the non-perpendicular line of vision of the microscope.

6.6. Fluorescence microscopy

Fluorescence images were acquired with an oil objective PlApo ×60 (N.A. = 1.4) on an Olympus IX70 microscope (Japan) connected to a FV500 (Fluoview) laser scanning confocal microscope. Excitation was performed using an Argon laser at 488 nm. Emitted light was collected through a BA 520–550 nm filter (for observing mainly NBD-PE fluorescence) and BA 660IF (observing the PDA fluorescence).

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